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(54) Title: REGULATION OF Apob FOR DIAGNOSIS, TREATMENT AND DRUG SCREENING FOR CARDIOVASCULAR AND METABOLIC DISORDERS OR SYNDROMES

(57) Abstract: The present invention involves a method of exploiting a novel apolipoprotein-B (apoB) degradation pathway to regulate plasma levels of apoB to treat cardiovascular or metabolic disorders or syndromes, a method of exploiting a novel apoliprotein-B (apoB) degradation pathway to diagnose cardiovascular or metabolic disorders or syndromes, a method of exploiting a novel apolipoprotein-B (apoB) degradation to screen for drugs to treat cardiovascular or metabolic disorders or syndromes, and a method of exploiting a novel apoliprotein-B (apoB) degradation pathway to screen for genes for diagnosing cardiovascular or metabolic disorders or syndromes.

REGULATION OF ApoB FOR DIAGNOSIS, TREATMENT AND DRUG SCREENING FOR CARDIOVASCULAR AND METABOLIC DISORDERS OR SYNDROMES

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims priority under 35 U.S.C. § 119 based upon U.S. Provisional Patent Application No. 60/161,537 filed October 26, 1999.

GOVERNMENT RIGHTS IN THE INVENTION

This invention was made with government support under grants DK 50376, HL 58541, HL 22263, HL 38956, HL 58884 awarded by the National Institutes of Health. The government has certain rights in the invention.

FIELD OF THE INVENTION

The present invention generally relates to the fields of cardiology and internal medicine and to a method of diagnosing and treating cardiovascular or metabolic disorders or syndromes, as well as a method of screening for drugs to treat such disorders or syndromes. More particularly, the present invention relates to a method of exploiting a novel apolipoprotein-B (apoB) degradation pathway to regulate plasma levels of apoB to treat cardiovascular or metabolic disorders or syndromes, to a method of exploiting a novel apolipoprotein-B (apoB) degradation pathway to diagnose cardiovascular or metabolic disorders or syndromes, to a method of exploiting a novel apolipoprotein-B (apoB) degradation pathway to screen for drugs to treat cardiovascular or metabolic disorders or syndromes, and to a method of exploiting a novel apolipoprotein-B (apoB) degradation pathway to screen for genes for diagnosing cardiovascular or metabolic disorders or syndromes.

BACKGROUND OF THE INVENTION

Apolipoprotein B (apoB), the major protein of atherogenic lipoproteins, is synthesized primarily by hepatic and intestinal cells. Most studies have focused on apoB metabolism in the liver, given the greater contribution to the plasma apoB pool made by that organ and the availability of relatively convenient primary and transformed hepatic cell models. ApoB message level and translational rate in hepatic cells are largely constitutive, and so secretory control is achieved primarily through co and post-translational degradation of the protein (e.g., see (2,3) for recent reviews).

Two specific mechanisms for the destruction of newly synthesized apoB in hepatic cells have been characterized. The first is endoplasmic reticulum-associated degradation (ERAD). Newly synthesized apoB in the endoplasmic reticulum (ER) is initially complexed with small amounts of lipid that is shuttled to the apoB by the microsomal triglyceride transfer protein (MTP) (4). During conditions of severe lipid deprivation (5,6) or MTP deficiency (7,8), this initial lipidation fails, and the apoB becomes ubiquitinylated, which targets it for degradation by proteasomes (9-13).

The second mechanism for degradation of newly synthesized apoB is the re-uptake pathway. Re-uptake occurs after fully assembled apoB-containing particles have been exported across the plasma membrane, but before they have diffused away from the vicinity of the cell by traversing the unstirred water layer that is adjacent to the plasma membrane (14) (see also (15,16)). A substantial percentage of these nascent apoB-containing particles bind cell-surface receptors, such as LDL receptors (14) or specific heparan sulfate proteoglycans (17-19), that then bring them back into the cell. Delivery to lysosomes and proteolytic degradation follows. The pathway is stimulated by sterol deprivation (14), which induces LDL receptor expression (20), or by the presence of molecules that can bridge between apoB-containing particles and cell-surface proteoglycans (21,22). The architecture of the liver may favor re-uptake, owing to the presence of diffusional impediments, such as the space of Disse and the fenestrated endothelial barrier, through which nascent lipoproteins must pass before escape into the circulation. Because apoB and cell-

surface lipoprotein receptors share portions of the secretory pathway, it is likely that binding occurs within the cell as well (14,16).

A large number of other agents or conditions have also been reported to reduce net output of apoB by stimulating intracellular degradation, such as severe choline deficiency (23), acute stimulation with insulin (24), and administration of long-chain polyunsaturated fatty acids with a double bond in the n-3 position, known as Ω-3 or fish oil fatty acids (25). Importantly, roles for the two known degradative pathways, ERAD and re-uptake, have not been reported in these circumstances.

In the current study, the mechanism of action of Ω-3 fatty acids was the focus. Consumption of these molecules has been associated with a lipid-lowering effect *in vivo* (26) and reductions in heart disease (27,28). Two specific Ω-3 fatty acids, eicosapentaenoic acid (EPA, 20:5) and docosahexaenoic acid (DHA, 22:6), have been shown to stimulate the degradation of newly synthesized apoB by cultured hepatocytes and hepatoma cells (25,29). Prior evidence is more consistent with a role for re-uptake than ERAD: Ω-3 fatty acids stimulate LDL receptor expression (30), and experiments with rat hepatoma McArdle RH-7777 cell clones that express a range of truncated human apoB cDNA constructs have suggested that degradation induced by Ω-3 fatty acids is more pronounced for the most buoyant lipoproteins (29), which would experience the greatest diffusional impediments and contain the most apoE, a ligand for both LDL receptors and HSPGs.

Therefore, the present invention involves examination of four items: the target for Ω-3–stimulated degradation, focusing on early *versus* fully assembled lipoprotein particles; the role of ERAD, focusing on a possible role for MTP inhibition and the participation of proteasomes; the role of re-uptake, focusing on LDL receptors, cell-surface heparan sulfate proteoglycans, and lysosomes; and intracellular localization and signaling involved in Ω-3–stimulated degradation. Based on these characteristics, the current studies indicate that Ω-3 fatty acids stimulate the degradation of newly synthesized apoB through a novel, third pathway that is distinct from either ERAD or re-uptake.

It is critical to define the metabolic pathway or pathways that are involved in hepatic overproduction of lipoprotein particles that lead to or are involved in

atherosclerosis, other cardiovascular disorders, and metabolic syndromes or disorders. There is a need for new and improved methods of diagnosis and treatment of patients at increased risk of atherosclerosis and of patients having any one of a variety of cardiovascular disorders or metabolic disorders or syndromes. Additionally, there is need for further identification of therapeutic targets for successful treatment of cardiovascular or metabolic disorders or syndromes and for testing of therapeutic agents directed against those targets. Current methods of treatment for cardiovascular disorders or metabolic disorders or syndromes involve the use of a variety of compounds, many of which can have undesirable side effects.

The atherosclerosis-causing lipoproteins are those containing apoB and are predominately made and secreted by the liver of mammals. The underlying molecular bases of apoB production under physiologic circumstances has been poorly understood. However, it has been shown that apoB-lipoprotein production is regulated largely by changes in the intracellular pre-secretory degradation of apoB. In other words, any factor or process that increases apoB degradation before it leaves the cell will reduce the net secretion of apoB-lipoproteins. By studying the effects of the fatty acids contained in fish oils (EPA, DHA), a key apoB degradation pathway has been uncovered.

The exploitation of this novel apoB degradation pathway to lower plasma concentration of apoB is an improvement over existing methods of regulating or modulating apoB plasma levels. For example, regulation of apoB levels by stimulating degradation of apoB by endoplasmic reticulum-associated degradation (ERAD) leads to a toxic accumulation of proteins intracellularly in the hepatocyte as the proteosomes become preoccupied with apoB degradation fail to degrade other proteins normally targeted for proteosomal degradation. The novel apoB degradation pathway of the present invention also provides a new avenue for effective diagnosis of a variety of cardiovascular disorders and of a variety of metabolic disorders or syndromes. Additionally, the novel apoB degradation pathway of the present invention also provides a new means of identifying compounds or therapeutics that successfully treat a variety of cardiovascular disorders and a variety of metabolic disorders and syndromes.

The novel apoB degradation pathway of the present invention has the following features that are novel and distinct from other well-characterized apoB degradative pathways: 1) apoB that is associated with large, lipid-rich lipoproteins is preferentially degraded in the pathway of the present invention compared to apoB associated with more dense, lipid-poor lipoproteins; and 2) this degradation occurs after apoB exits the endoplasmic reticulum (ER) and is not inhibited by compounds known to inhibit ER-associated apoB degradation, such as proteosomal inhibitors.

Additionally, the degradation pathway of the present invention is inhibited by inhibitors of PI-3-kinase (such as wortmannin), an enzyme that is part of metabolic signaling cascades, including one stimulated by insulin. Insulin has been shown by others to preferentially stimulate the degradation of apoB associated with lipid-rich lipoproteins. Insulin-stimulated apoB degradation was inhibited by wortmannin. Therefore, the degradation pathway stimulated by EPA and DHA is the same as that stimulated by insulin, given the identical lipoprotein particle preference and the response to wortmannin. Previously, work on the insulin-stimulated pathway has not led to an appreciation that this pathway is distinct from ER-associated destruction of apoB.

It is important to note that there is a genetic disorder, familial combined hyperlipidemia (FCHL), the most frequent genetic lipid disorder in those with premature atherosclerotic heart disease. These patients exhibit hepatic overproduction of apoB and many have insulin resistance. ApoB overproduction in this disorder is a result of a decrease in the activity of the degradation pathway regulated by EPA, DHA, and insulin.

From clinical turnover studies, it is also known that apoB production rates significantly vary among individuals in the general population. The activity of the degradation pathway will regulate apoB production even in those without FCHL.

Conceptually the present invention opens up major insights into apoB-lipoprotein metabolism. At a practical level, the assay system that has been established and validated in the present invention can be used to identify molecules and their associated DNA and RNA sequences that can be used for genetic epidemiological studies, diagnostic evaluation, and therapeutic manipulation

(including drug screening methods to identify agents that regulate the activity of this degradative pathway).

Previously it was shown that Ω-3 fatty acids reduce secretion of apolipoprotein B (apoB) from cultured hepatocytes by stimulating post-translational degradation. In this report, this process is now characterized, particularly in regard to the two known processes that degrade newly synthesized apoB, ER-associated degradation and re-uptake from the cell surface. First, it was found that large, apparently fully assembled apoB-lipoprotein particles are the target of Ω-3–induced degradation, and apoB polypeptide length is not a determinant. Second, based on several experimental approaches, ER-associated degradation is not involved. Third, re-uptake, the only process known to destroy fully assembled nascent lipoproteins, was clearly active in primary hepatocytes, but Ω-3–induced degradation of apoB continued even when re-uptake was blocked. Trapping apoB in the ER by adding Brefeldin A inhibited Ω-3 fatty acid-induced degradation, indicating a post-ER process. To determine the signaling involved, wortmannin, a PI-3 kinase inhibitor, was used and blocked much of the Ω-3 fatty acid effect. Therefore, nascent apoB is subject to ERAD, re-uptake, and a third distinct degradative pathway that involves a post-ER compartment and PI-3 kinase signaling. Physiologic, pathophysiologic, and pharmacologic regulation of net apoB secretion involve alterations in any of these three degradative steps.

SUMMARY OF THE INVENTION

Accordingly, it is an object of the present invention to exploit a novel apoB degradation pathway to regulate plasma levels of apoB to treat cardiovascular and metabolic disorders or syndromes.

It is another object of the present invention to exploit a novel apoB degradation pathway to diagnose cardiovascular or metabolic disorders or syndromes.

It is yet another object of the present invention to exploit a novel apoB degradation pathway to screen for drugs to treat cardiovascular or metabolic disorders or syndromes.

BRIEF DESCRIPTION OF THE DRAWING

FIG. 1. DHA inhibits the secretion of $apoB_{48}$ on VLDL, while denser $apoB_{48}$-containing particles are relatively unaffected. Rat primary hepatocytes were incubated at 37°C for 4 h with either OA or DHA (0.8 mM, complexed to 0.16 mM BSA) in the presence of [^{35}S]methionine. Conditioned media samples were subjected to density gradient fractionation, and the labeled $apoB_{48}$ content of each density class was determined by immunoprecipitation followed by SDS-PAGE and then scintillation counting of the excised gel bands containing $apoB_{48}$. Results shown are the mean ± SE (n=6).

FIG. 2. DHA inhibits the secretion of $apoB_{100}$ on VLDL, while denser $apoB_{100}$-containing particles are relatively unaffected. HepG2 cells were treated and their conditioned media samples analyzed as in **Fig. 1**, except that the excised gel band contained $apoB_{100}$. Results shown are the mean ± SE (n=6).

FIG. 3. Ω-3 fatty acids inhibit the secretion of total labeled apoproteins associated with VLDL. Rat primary hepatocytes were incubated 4 h with OA, EPA, or DHA (0.8 mM, complexed to 0.16 mM BSA) or with BSA alone (0.16 mM) in the presence of [^{35}S]methionine. Conditioned media samples were subjected to density gradient fractionation and the total labeled apoprotein contents of the VLDL fractions were quantified by TCA precipitation followed by scintillation counting. After normalization to mg of cell protein, the secretion of labeled apoproteins in the presence of the fatty acid/BSA complexes relative to the secretion in the presence of BSA alone was calculated. Results shown are the mean ± SE (n=6).

FIG. 4. Effects of EPA or DHA on the secretion of different labeled apoprotein species associated with VLDL. Rat primary hepatocytes were treated as in **Fig. 3**. Conditioned media samples were subjected to density gradient

fractionation to isolate VLDL, and the labeled apoprotein species were separated by SDS-PAGE. The resulting fluorogram shows the results from duplicate wells. The migration of the apoprotein size standards is indicated on the right.

FIG. 5. Ω-3 lipids are good substrates for MTP-mediated lipid transfer. HepG2 cells were incubated for 14 h at 37°C with ^{14}C-labeled oleate (OA) or ^{14}C -labeled EPA complexed to BSA (final concentration of either fatty acid was 0.8 mM). Total lipids were extracted from the washed cell monolayers and separated by preparative TLC into triglyceride (TG), cholesteryl ester (CE), phosphatidylcholine (PC), and phosphatidylethanolamine (PE). The isolated lipid classes were then incorporated (to a content of 0.5 mol%) into vesicles that were used as donors in lipid transfer assays with recombinant MTP. Results were normalized to the transfer of [^{14}C]triolein and are shown as mean values ± SE (n=4).

FIG. 6. Ω-3 lipids do not inhibit the transfer of non-Ω-3 lipids by MTP. Artificial donor vesicles were prepared in which PC that contained oleate esterified at the sn-2 position was replaced with an increasing mole fraction of PC that contained DHA or EPA groups. The transfer by purified bovine MTP of either labeled triolein or labeled POPC to acceptor vesicles was measured in a lipid transfer assay. Results shown are the amounts of labeled lipids transferred relative to the transfer observed with donor vesicles containing no Ω-3 acyl chains, and are expressed as mean values +/- SE (n=4).

FIG. 7. Inhibition of MTP reduces the secretion of newly synthesized apoB$_{100}$ without affecting apoE. Rat hepatoma cells were incubated at 37°C for 4 h with [^{35} S]methionine in the absence (Control) or presence (MTPI) of an inhibitor of MTP activity. Equal aliquots of the conditioned media samples from duplicate wells were subjected to separate immunoprecipitations with anti-apoB or anti-apoE antiserum. For each well, the resulting pellets from the two immunoprecipitations were combined and analyzed by SDS-PAGE and fluorography.

FIG. 8. EPA stimulates apoB degradation even when proteasomes are inhibited. Rat hepatoma cells were incubated at 37°C for 4h in [^{35} S]methionine-containing medium supplemented with either BSA (C) or EPA/BSA complexes

(EPA) in the absence (EPA) or presence (EPA+LAC) of the proteasome inhibitor, lactacystin. Samples of cell lysates and conditioned media were subjected to immunoprecipitation analysis with anti-apoB antiserum, followed by SDS-PAGE and fluorography. In three separate experiments, the effect of lactacystin on EPA-induced degradation averaged less than 20%.

FIG. 9. DHA inhibits the secretion of newly synthesized VLDL-apoproteins even when re-uptake is blocked. Rat primary hepatocytes were treated as in **Fig. 1**, except that media in the indicated wells was also supplemented with heparin (10 mg/ml). Total labeled VLDL-apoproteins were determined as in **Fig. 3**. The results for the fatty acids are expressed relative to the corresponding result for BSA and are shown as mean $\pm$ SE (n=6).

FIG. 10. Brefeldin A increases the total recovery of newly synthesized $apoB_{100}$ from DHA-treated cells. Rat hepatoma cells were incubated at 37°C for 4 h in [^{35}S]methionine-containing medium supplemented with either DHA complexed to BSA (DHA) or DHA/BSA plus 4 mg/ml BFA (DHA+BFA) The total recovery of labeled $apoB_{100}$ was determined by immunoprecipitation of cell lysates and media samples, followed by SDS-PAGE and phosphorimager analysis. The data were normalized to total labeled protein in cell lysate plus media, determined using TCA precipitation. The results shown are mean $\pm$ SE (n=4).

FIG. 11. Wortmannin increases the recovery of apoB mass secreted from DHA-treated cells. Rat primary hepatocytes were incubated 5 h in medium containing either DHA/BSA (DHA) or DHA/BSA plus 1 μM wortmannin (DHA+Wort). The recovery of apoB mass in the conditioned medium samples was determined by radioimmunoassay. Results shown are the mean $\pm$ SE (n=5).

Fig. 12. A schematic diagram of two previously known mechanisms for destruction of newly synthesized hepatocyte apoB. These methods of degradation are ERAD and re-uptake lysosomal destruction.

DETAILED DESCRIPTION OF THE INVENTION

Previously it was reported that ω3 fatty acids (FA) reduce secretion of apoB from cultured hepatocytes by ≈50% through stimulation of post-translational degradation (JCI 91:1380). The process is now characterized.

First, the substrate was examined. Addition of ω3 FA to rat primary hepatocytes or human hepatoma-G2 cells reduced secretion of large VLDL-like particles, with no effect on apoB in the HDL density range. This pattern was independent of apoB species ($apoB_{100}$, $apoB_{48}$). Other components of nascent VLDL, such as apoE and apoCs, were also degraded. Thus, large, fully assembled lipoprotein particles are the target, and apoB length is not a determinant.

Second, it was necessary to examine the role of ER-associated degradation (ERAD), an early step mediated by proteosomes (JBC 272:20427) and provoked by incomplete initial lipidation of apoB by microsomal transfer protein (MTP). ω3 lipids were good substrates for MTP and did not block transfer of other lipids. Inhibitors of MTP reduced VLDL and HDL apoB, with no effect on apoE, i.e., a pattern unlike ω3 FA effects. Proteosome inhibitors failed to block ω3 FA-mediated degradation of apoB, excluding a role for ERAD.

Third, it was necessary to examine re-uptake, the sole process known to destroy fully assembled nascent particles. Addition of heparin to block re-uptake via proteoglycans or LDL receptors (JBC 267:13284) doubled VLDL apoB secretion, as expected, but ω3 FA still reduced VLDL output by ≈50%. Inhibitors of lysosomes, the destination of re-uptake, had no effect.

Thus, the ω3 FA effect is distinct from ERAD and from re-uptake. To pinpoint the site of the ω3 FA effect, we used Brefeldin A (BFA) to trap apoB in the ER. BFA completely blocked ω3 FA-induced apoB degradation, indicating that ω3 FA act post-ER, in the Golgi. To determine the signaling involved, we used Wortmannin, a PI-3 kinase inhibitor, which blocked most of the ω3 FA effect.

Therefore, nascent apoB is subject to ERAD, re-uptake, and a third distinct degradative pathway, which involves a post-ER compartment and PI-3 kinase

signaling. Physiologic, pathophysiologic, and pharmacologic regulation of net apoB secretion involves alterations in any of these three degradative steps.

Additional data for the triple threat to nascent apolipoprotein-B: evidence for multiple, distinct degradative pathways

Methods

Male Sprague-Dawley rats (Ace Animals, Boyertown, PA) weighing 200-225 g were used to obtain hepatocytes by a protocol approved by the institutional animal care committee. All reagents, unless otherwise specified, were purchased from Sigma (St. Louis, MO). [^{14} C]eicosapentaenoic acid, [^{35} S]methionine, and [^{14}C]triolein, and Enhance solution were purchased from New England Nuclear (Boston, MA); [^{14} C]oleic acid and 1-palmitoyl-2-[^{14} C]oleoyl phosphatidyl choline (POPC) were purchased from Amersham (Arlington Heights, IL). Immunoprecipitin (staph A cells) was purchased from Bethesda Research Labs (Gaithersburg, MD). Collagenase was purchased from Worthington Biochemical (Freehold, NJ). Rat hepatoma (McArdle RH-7777) and human hepatocarcinoma (HepG2) cells were purchased from American Type Tissue Collection (Manassas, VA). Rabbit polyclonal antisera to rat apoB or apoE and mouse monoclonal antibody to rat apoB were developed in the laboratories and were previously described (25,31,32).

Cell Culture Techniques

Rats fed ad-libitum were sacrificed in the morning, and liver cells were isolated by collagenase perfusion using 0.225 mg collagenase/ml dissolved in Krebs-Ringer buffer containing 1.66 mM calcium. Hepatocytes were purified by differential centrifugation through a 45% Percoll solution, and their viability was determined by exclusion of ethidium bromide stain, using a fluorescence microscope to visualize the stained nuclei of damaged cells. Only preparations with >90% intact cells were used.

Cells were plated at a density of 2 X 10^6 cells/ml on 60-mm culture dishes (previously coated with poly-D-lysine) in modified M199 medium (M199, 1% fetal bovine serum [FBS], 1 mM nicotinamide, 0.1 nM insulin, 3 mg/ml choline, 1.1% L-glutamine, 1% BSA). After a four-hour attachment period, the medium was changed (modified M199 identical to the above except BSA was omitted and the concentration of FBS was raised to 10%). The next morning, cells were washed in serum-free medium three times, the experimental media added, and the cells incubated at 37°C for 4-6 hours. The experimental medium consisted of serum-free RPMI containing the appropriate isotopes (see below) and fatty acids (FA) present at a final concentration of 0.8 mM complexed to BSA (FA:BSA molar ratio = 5:1). The fatty acids used were oleic (OA), EPA, and DHA. Control medium was identical, except that BSA (0.16 mM) without fatty acids was present. For metabolic labeling of proteins, [^{35} S]methionine (70 uCi/ml) was included in the experimental media.

As indicated in Results, in some experiments, McArdle RH-7777 (rat hepatoma) or HepG2 (human hepatocarcinoma) cells, maintained as in (14,29), were substituted for the rat primary hepatocytes.

Secretion of Newly Synthesized Apoproteins

Following the 4-6 hour incubation period, the cell monolayer was washed, then solubilized in 0.1M NaOH and the cell protein determined by the Lowry method (33). Each 60 mm dish contained approximately 1-1.5 mg of cell protein. The secreted apoproteins were isolated from the medium either by ultracentrifugation or immunoprecipitation. To isolate the $d < 1.006$ (VLDL) and $1.006 < d < 1.063$ (IDL and LDL) g/ml density classes by centrifugation, sequential 20 hour runs at 48,000 rpm in a 50Ti Beckman rotor at 4°C were performed. After the first run at $d = 1.006$, the upper VLDL layer was harvested and the density of the infranatant adjusted to 1.066 g/ml with solid KBr. After being overlaid with a KBr solution of density 1.063, the adjusted infranatant was re-centrifuged as before to isolate the IDL and LDL fractions. In some experiments, the HDL fraction ($1.063 < d < 1.21$) was isolated by sequential density centrifiugation. Lipoprotein

fractions were then dialyzed against 0.9% NaCl containing 10 mM unlabeled methionine, 0.2 mM PMSF, and 2 mM EDTA. Dialyzed lipoproteins were delipidated using 9 volumes of 100% isopropanol. Apoproteins were then collected by centrifugation in a Sorval HB-4 rotor at 10,000 rpm and 4°C for 20 min, and dissolved in buffer (0.01 M phosphate, pH 7, 1% SDS, 10% glycerol), in preparation for electrophoresis.

To immunoprecipitate apoB or apoE from unfractionated conditioned medium, monospecific rabbit antiserum to rat apoB or apoE was used. Briefly, an aliquot of the medium was mixed with an equal volume of diluted antiserum (1:100 in PBS and 1% BSA) and incubated overnight at 4°C. Staph A was added in the form of Immunoprecipitin, and the resultant precipitate containing the immune complexes was washed extensively, the staph A cells removed, and the isolated apoprotein dissolved in gel sample buffer (0.0625M tris/Cl, pH 6.8, 20% glycerol, 2% SDS). Electrophoretic separation of apoVLDL, apoIDL/LDL, and immunoprecipitates was accomplished using 3.5% acrylamide-18% glycerol gels as described (34). An aliquot of each sample was taken for scintillation counting, so that total radioactivity applied to each lane could be determined. Also, protein size standards were included in each gel.

After electrophoresis, the gels were stained, fixed, soaked in Enhance fluor solution, and dried following protocols supplied by the manufacturer. Dried gels were then exposed to X-ray film at -70°C and signals on the resulting fluorograms were typically quantified by densitometry or phosphorimaging. Using the total radioactivity applied to each lane of the gel and the relative signal intensity of each band in that lane, the radioactivity associated with each apoprotein species was calculated. In some cases, gel bands were excised and the incorporated radioactivity measured directly by scintillation counting. As a control, the incorporation of [^{35}S]methionine into total cellular and secreted proteins was measured by TCA-PTA precipitation, as previously described (25).

To determine the differential effects of MTP inhibition on the secretion of labeled apoB and apoE, in some experiments, McArdle RH-7777 cells were pre-treated for 30 min with MTP inhibitor BMS-200150 (10 mM dissolved in DMSO;

(35)) or DMSO alone (final concentration 0.5%) before adding radiolabel. The cells were further incubated for 4 h and the conditioned media contents of apoB and apoE determined by immunoprecipitation and SDS-PAGE analysis as described above.

To determine the effects of inhibiting PI3-kinase on apoB secretion, rat primary hepatocytes were pre-treated for 30 min with 1 μM wortmannin (dissolved in DMSO) or DMSO alone (final concentration 0.5%), which were maintained throughout the experiment. DHA/BSA complexes were then added and the cells were further incubated for 4 h. The apoB contents of the conditioned media samples were determined by radioimmunoassay (31). In some experiments, [^{35}S]methionine was used to pulse label apoB. The effect of wortmannin on labeled apoB recovery from cell lysate and conditioned medium at the 15 min and 90 min time points of the chase period was determined by immunoprecipitation and SDS-PAGE analysis (25).

In some experiments, the protein trafficking inhibitor Brefeldin A (BFA) was used to assess whether Ω-3 fatty acids affected event(s) post-ER. Rat hepatoma cells were incubated at 37°C for 4 h in [^{35}S]methionine-containing medium supplemented with either DHA complexed to BSA (DHA/BSA) or DHA/BSA plus 4 mg/ml BFA. The total recovery of labeled $apoB_{100}$ was determined by immunoprecipitation of cell lysates and media samples, followed by SDS-PAGE and phosphorimager analysis.

To determine whether the proteasome mediated Ω-3 fatty acid-induced apoB degradation, rat hepatoma cells were incubated at 37°C for 4h in [^{35}S]methionine-containing medium supplemented with either BSA or EPA/BSA complexes in the absence or presence of the proteasome inhibitor, lactacystin (10 mM; purchased from the laboratory of Dr. E.J. Corey, Harvard University). Samples of cell lysates and conditioned media were subjected to immunoprecipitation analysis with anti-apoB antiserum, followed by SDS-PAGE and fluorography. To determine whether Ω-3 fatty acids targeted apoB to lysosomal degradation, a similar experiment was performed substituting ammonium chloride (40 mM) for lactacystin. At this concentration of ammonium chloride, there was no evidence of cell toxicity as assessed by TCA-PTA precipitation analysis, but there was >80% inhibition of the

degradation of exogenously added 125 I-LDL (a generous gift of Dr. Ira Tabas, Columbia University).

Effects on Nascent VLDL Re-Uptake

To evaluate whether there were differential effects of fish oil fatty acids and OA on the re-uptake of newly synthesized VLDL, an experimental design identical to that described above was employed, except heparin (0.1 mg/ml or 10 mg/ml) was added to the treatment media to block LDL receptor-dependent and proteoglycan - mediated re-uptake (17). Control experiments were performed showing that the flotation properties of VLDL were unchanged in the presence of heparin.

Effects on Microsomal Triglyceride Transfer Protein Activity

The effects of the various treatments on the activity of microsomal triglyceride transfer protein (MTP) were measured at the end of the incubations by a fluorescent assay procedure (using a reagent kit from Roar Biomedical, NY). To determine whether the lipid classes synthesized in the presence of OA or Ω-3 fatty acid were equivalent substrates for MTP, either HepG2 cells or primary hepatocytes were incubated with radiolabeled OA or EPA. After isolation of total cellular lipids by isopropanol extraction, lipid classes (containing the endogenously labeled cholesteryl ester, triglyceride, and phospholipids) were resolved and collected by preparative thin layer chromatography. Each lipid class was incorporated into separate donor vesicles, which were added to a transfer reaction mixture containing acceptor vesicles and purified bovine MTP (50 μg). MTP-mediated transfer of labeled lipids from donor to acceptor vesicles was then measured, as in reference (36), which contains a complete description of the materials, conditions, and analytical procedures related to this vesicle-based MTP assay.

Statistical Analysis

Results are displayed as mean ± SE, n ≥ 3. Absent error bars in figures indicate SE values smaller than the drawn symbols. For comparisons between a single experimental group and a control, the unpaired, two-tailed t-test was used. For comparisons involving several groups simultaneously, analysis of variance (ANOVA) was initially used. When the ANOVA indicated differences amongst the groups, pairwise comparisons of each experimental group *versus* the control group were performed using the Bonferroni test. Analyses were performed with the InStat programs (GraphPAD Software, San Diego, CA).

Results

Nature of the target for Ω-3 fatty acid-induced degradation:

Prior studies using rat hepatoma McArdle RH7777 (McA) cell clones that express a range of artificially truncated human apoB constructs have suggested that buoyant lipoproteins, regardless of the precise length of the transfected apoB construct, are the most susceptible to degradation induced by Ω-3 fatty acids (25,29). Two approaches were used to test this suggestion with native, rather than artificially truncated, forms of apoB. First, rat primary hepatocytes were studied. This cell type is a non-transformed cell that secretes $apoB_{100}$ almost exclusively in the form of VLDL, while dividing its production of $apoB_{48}$ between particles with the density of VLDL (~2/3 of secreted $apoB_{48}$) and HDL (~1/3 of secreted $apoB_{48}$) (37).

Rat primary hepatocytes were incubated at 37°C for 4 h with OA or DHA complexed to BSA (or BSA alone) in the presence of [^{35}S]methionine, and the conditioned media were separated into VLDL and HDL fractions by density gradient ultracentrifugation. The content of labeled $apoB_{48}$ in each density fraction was then quantified. As shown in **Fig. 1**, the recovery of labeled $apoB_{48}$ from the VLDL fraction of conditioned media from cells treated with DHA averaged only ~35% ($P<0.001$) compared to the results with samples from the OA group,

whereas the recovery of labeled $apoB_{48}$ from the HDL fraction was essentially independent of treatment. Thus, a single type of apoB, $apoB_{48}$, was differentially affected by DHA depending entirely on the density of the associated lipoprotein.

To pursue the implication that the apoB sequence *per se* is not a determinant of Ω-3 fatty acid-induced degradation, a different cell culture model was utliized, the human hepatocarcinoma HepG2, which produces $apoB_{100}$, but no $apoB_{48}$. A small, but easily measurable amount of HepG2 $apoB_{100}$ is secreted as part of lipoproteins with the density of VLDL, with the majority appearing in the denser IDL/LDL ($1.006 < d < 1.063$ g/ml) and HDL ($1.063 < d < 1.21$) fractions. In previous studies (38), it was found that EPA or DHA treatment significantly reduced the secretion of newly synthesized VLDL-$apoB_{100}$ by HepG2 cells, but the effects on the denser apoB-lipoproteins were not examined. Therefore, HepG2 cells were incubated 4 h with [^{35} S]methionine and either OA or DHA, and the conditioned media were separated by centrifugation into fractions of $d < 1.006$ g/ml (VLDL) and $1.006 < d < 1.21$ g/ml (LDL+HDL). The recoveries of apoB100 from each density fraction are summarized in **Fig. 2**.

As expected, a small amount of labeled $apoB_{100}$ was secreted into the VLDL fraction in either treatment group (**Fig. 2**, left 2 bars; note the difference in the scale of the left and right Y-axes). Nevertheless, the *relative* recovery of newly secreted VLDL-$apoB_{100}$ was considerably lower after DHA treatment (~35% of that in the OA group; $P < 0.01$, consistent with **Fig. 1**). In contrast, DHA treatment only mildly affected the relative recovery of labeled $apoB_{100}$ from the higher density LDL+HDL class (~75% of that in the OA group; right 2 bars). Overall, the separate results from the primary hepatocytes, in which $apoB_{48}$ appears in different density fractions, and HepG2 cells, in which $apoB_{100}$ also appears in different density fractions, clearly demonstrate that it is a property of the lipoprotein particle, not the primary amino acid sequence of apoB, that is a critical factor in determining susceptibility to degradation induced by Ω-3 fatty acids. Similar results have been obtained in rat hepatoma McA cells clones expressing a range of artificially truncated human apoB constructs (29). These previous and current results suggest a robust phenomenon independent of the primary sequence of apoB.

An important implication of this conclusion is that a given apoB molecule must be part of a buoyant lipoprotein particle before that molecule is degraded by the process induced by Ω-3 fatty acids. The simplest way for this to occur is if the lipoprotein particles are fully or nearly fully assembled before Ω-3 fatty acid-induced degradation. Of course, if entire VLDL particles are removed from the secretory pathway upon Ω-3 fatty acid treatment, then other VLDL apoproteins should also be affected. To test this prediction, it was necessary to examine whether there are concurrent changes in the secretion of newly synthesized VLDL-apoproteins other than apoB. Rat primary hepatocytes were treated with BSA, OA, or Ω-3 fatty acids in the presence of [^{35} S]methionine, and the conditioned media collected. After centrifugation to isolate the $d < 1.006$ density fraction, VLDL samples were delipidated and the incorporation of radiolabel into apoproteins was determined.

As shown in **Fig. 3**, the secretion of total labeled VLDL-apoproteins was significantly reduced in the Ω-3 fatty acid treated groups relative to the results from the BSA and OA groups ($P < 0.001$). SDS-PAGE was used to separate the total apoproteins into the individual species. Visual inspection of the resulting fluorograms, such as the one shown in **Fig. 4**, indicated that treatment with DHA (or EPA) produced substantial decreases in the signal intensities of labeled $apoB_{100}$, $apoB_{48}$, apoE, and apoCs associated with nascent VLDL. The combined numerical analysis from repeated experiments is summarized in **Table 1**, except that the results for the apoCs were not included, because such a small fraction (<5% in any lane) of radioactivity was attributable to these apoproteins, consistent with their being a minor componnent of VLDL secreted by rat hepatocytes (37). The results with apoE are particularly informative, because this apoprotein is known to be added to VLDL particles relatively late in the lipoprotein assembly process (39-41). Thus, Ω-3 fatty acids induce a global loss of all VLDL-associated apoproteins, including apoE, implying a late effect in the secretory pathway that occurs after the lipoproteins are completely or nearly completely assembled.

Table 1: The secretion of newly synthesized VLDL-apoproteins by rat hepatocytes incubated with fatty acid/BSA complexes, expressed as the percentage of secretion observed with BSA alone.

Fatty acid	ApoE	$ApoB_{48}$	$ApoB_{100}$
OA	84 ± 3	132 ± 11	96 ± 4
EPA	45 ± 1	45 ± 3	25 ± 2
DHA	53 ± 2	39 ± 3	24 ± 3

Rat hepatocytes were incubated for 6 h with [^{35}S]methionine in the presence of BSA or the indicated fatty acid/BSA complexes. VLDL fractions were isolated from conditioned media by ultracentrifugation, delipidated, and the labeled apoprotein species were separated by SDS-PAGE. The incorporated radioactivity (dpm) in each apoprotein band was determined by excision of the band and scintillation counting. The data were then normalized to mg of cell protein. The result for each apoprotein is the mean ± SE (n=6) of the ({dpm recovered after fatty acid incubation}÷{dpm recovered after BSA incubation}) X 100. Compared to the results in the OA group, there were significant ($P<0.0001$) reductions in $apoB_{100}$, $apoB_{48}$, and apoE in either the EPA or DHA group.

Relationship between Ω-3 fatty acid-induced apoB degradation and ERAD

ApoB-lipoprotein biogenesis involves an early, regulated degradative process that is mediated by proteasomes (9-11), associated with the endoplasmic reticulum (13), and provoked by inadequate MTP-mediated initial lipidation of newly synthesized apoB (11,12). Although the data point towards events later in the secretory pathway, it was nevertheless necessary to directly examine if Ω-3 fatty acids act at this early step.

First, it was necessary to test the possibility that Ω-3 fatty acids stimulate apoB degradation by impeding MTP-dependent early lipidation, thereby targeting apoB to proteasomes. Rat primary hepatocytes were pre-treated for 6 h with BSA, OA, or EPA, and then the MTP activity in lysates of these cells was assessed using artificial donor and acceptor vesicles, with [^{14}C]triolein as the MTP substrate, as described (36). No difference in MTP-mediated transfer of labeled triolein was seen in the different cell lysates.

Nonetheless, it is still possible that DHA- or EPA-enriched lipids are poor substrates for MTP-mediated transfer, especially because MTP activity has been reported to exhibit some dependence on the fatty acyl composition of its lipid substrates (36). An intracellular abundance of relatively poor lipid substrates, produced during incubations of hepatocytes with EPA or DHA, could be functionally equivalent to MTP inhibition. Thus, rat primary hepatocytes and HepG2 cells were incubated with radiolabeled OA or EPA to allow incorporation of these fatty acids into lipid esters. After extraction of total cellular lipids, triglycerides, cholesteryl ester, and phospholipids were each isolated by preparative TLC and reconstituted into donor vesicles for a lipid transfer assay, using purified bovine MTP (Methods). As shown in **Fig. 5**, there was no decrease in the ability of MTP to transfer any of the lipid species upon enrichment with Ω-3 fatty acyl groups. Similar results were also obtained with lipids derived from rat primary hepatocytes treated with labeled OA or EPA for 6 h.

Another approach to detect the effects of Ω-3 fatty acid enrichment on MTP-mediated lipid transfer was to determine if lipids containing Ω-3 fatty acyl groups

inhibit the transfer of non-Ω-3 lipids. A totally defined system was prepared, using artificial donor vesicles composed of unlabeled phosphatidylcholine plus [^{14}C]triolein or 1-palmitoyl-2-[^{14}C]oleoyl phosphatidylcholine (POPC). As above, the source of MTP was a purified bovine preparation (Methods). MTP-mediated transfer of the labeled lipids from these vesicles to an artificial acceptor was then measured. To test the effects of lipids containing Ω-3 fatty acyl groups, different compositions of unlabeled phosphatidylcholine in the donor vesicles were compared, from 0%-30% DHA or EPA groups at the sn-2 position *(i.e.,* 100%-70% oleate esterified at the sn-2 position). As shown in **Fig. 6**, no significant effects were found on the transfer of labeled triolein or labeled POPC, even when the % enrichment in Ω-3 fatty acids exceeded that achieved in hepatic cells incubated with EPA for 6 h (42). Overall, these data do not support the hypothesis that Ω-3 fatty acids interfere with the initial, MTP-dependent phase of VLDL assembly.

This conclusion is also supported by the pattern of apoprotein secretion after MTP inhibition. Upon treatment of rat hepatoma cells with BMS compound # 200150 (35), an inhibitor of MTP, the secretion of newly synthesized apoB and apoE was measured. As seen in **Fig. 7**, MTP inhibition almost completely abolished the secretion of $apoB_{100}$, as expected (35), but there was no significant effect on apoE secretion. This pattern is unlike the effects of Ω-3 fatty acids, which reduce the secretion of both apoproteins (**Fig. 4** and **Table 1**).

Further proof that Ω-3 fatty acids affect a step distal to MTP-dependent lipoprotein assembly comes from examining the role of the proteasome, which mediates apoB degradation after inhibition of either lipid synthesis or MTP-mediated lipid transfer *(e.g.,* (12,13)). Rat hepatoma cells were treated with either BSA or with EPA/BSA complexes in the absence or presence of the proteasomal inhibitor, lactacystin (Methods). Typical data are shown in **Fig. 8**, in which lactacystin produced little if any inhibition of EPA-induced degradation. Thus, involvement of MTP or ERAD cannot explain four key characteristics of Ω-3 fatty acid–induced degradation: it is specific to buoyant lipoproteins; it occurs without any inhibition of MTP; there are collateral casualties, such as apoE; and it is independent from proteasomes.

Relationship between Ω-3 fatty acid-induced degradation and re-uptake

To demonstrate re-uptake of nascent VLDL and to evaluate its potential contribution to the effects of Ω-3 fatty acids, rat primary hepatocytes were treated with either OA or DHA, with or without the addition of heparin to the culture medium. The concentration of heparin was 10 mg/ml, which blocks lipoprotein binding to both LDL receptors and HSPGs (reference (17) and citations therein). As shown in **Fig. 9**, the net secretion of newly synthesized VLDL-apoproteins during treatment with either OA or DHA was increased approximately two-fold ($P<0.01$) by the addition of 10 mg heparin/ml. Thus, there is substantial re-uptake of nascent VLDL by primary hepatocytes in the presence of either fatty acid. Nevertheless, blocking re-uptake with 10 mg heparin/ml did not affect the ability of DHA to reduce VLDL apoprotein output: secretion of VLDL apoproteins in the presence of DHA was approximately 50% of the OA control, independent of heparin treatment. Thus, re-uptake of newly exported apoB cannot explain the effect of Ω-3 fatty acids on lipoprotein secretion.

A similar experiment was conducted with a low concentration of heparin (0.1 mg/ml) that blocks lipoprotein binding to HSPGs without affecting LDL receptor binding (17). No "bridging molecules," such as lipoprotein lipase, were added to enhance lipoprotein-HSPG interactions. Under these conditions, the low concentration of heparin failed to increase apoB output in the presence of either fatty acid (*cf.* **Fig. 8** and accompanying text in reference (17)). Thus, under these specific conditions, re-uptake of nascent VLDL is substantial; it is mediated primarily by the binding of $apoB_{100}$ or apoE to the LDL receptor, without significant involvement of cell-surface HSPGs; and the inhibitory effect of Ω-3 fatty acids persists during blockage of re-uptake.

More evidence against the involvement of re-uptake in Ω-3 fatty acid-induced degradation of apoB is based on the knowledge that lipoproteins captured by either LDL receptors (20) or heparan sulfate proteoglycans (17,19) are directed to lysosomes. Thus, rat primary hepatocytes cells were treated with either OA or DHA in the absence or presence of ammonium chloride, a lysosomal inhibitor.

Under these conditions, ammonium chloride decreased the degradation of ^{125}I-LDL (Methods), but there was no decrease in DHA-induced degradation of newly synthesized apoB. Overall, involvement of re-uptake cannot explain two key characteristics of Ω-3 fatty acid–induced degradation: it is unaffected by even high concentrations of heparin, which blocks re-uptake of newly exported apoB via LDL receptors and HSPGs, and it is independent from lysosomes.

Intracellular localization and signaling involved in Ω-3–induced degradation of apoB

The ERAD-proteasome and re-uptake processes represent the initial and the final opportunities, respectively, for a hepatic cell to regulate the net secretion of apoB by targeting it to degradation. Because present data do not support a model in which Ω-3 fatty acids exert their effects at either of these steps, it must be presumed that they induce a distinct, third 'threat' to apoB at an intermediate site that is post-ER but before export across the plasma membrane. To test this possibility, rat hepatoma cells were treated with OA or DHA in the absence or presence of Brefeldin A (BFA), which blocks vesicular traffic from the ER to the Golgi. Thus, BFA treatment traps and isolates nascent apoB in the ER, which would be expected to protect apoB from post-ER degradation.

In preliminary studies performed in the absence of exogenous fatty acids, BFA did not affect apoB synthetic rate or total cellular protein synthesis, but did suppress total protein secretion, as expected. Next it was necessary to examin the recovery of labeled $apoB_{100}$ from the lysates and conditioned media from rat hepatoma cells that had been pre-treated with either DHA alone, or DHA supplemented with BFA. As shown in **Fig.10**, BFA substantially increased apoB recovery in the presence of DHA (by $\sim 50\%$; $P<0.01$). Thus, by preventing its exit from the ER, apoB was substantially protected from the Ω-3 fatty acid-induced degradation, indicating that the degradation occurred post-ER.

It was recently observed that BFA also protects apoB from acute insulin-stimulated degradation in rat primary hepatocytes (43), a process that depends on PI-3 kinase activation (43,44). Therefore, to determine if similar signaling is also involved in the effects of Ω-3 fatty acids, rat primary hepatocytes were treated with DHA in the absence or presence of wortmannin (1 mM), a PI-3 kinase inhibitor, for 5 h and then, by radioimmunoassay, apoB mass was measured that had accumulated in the conditioned medium (Methods). As shown in **Fig. 11**, apoB recovery increased by $\sim 50\%$ ($P < 0.01$) in the wortmannin-treated group, indicating that Ω-3 fatty acids act via an inducible process involving PI-3 kinases. Wortmannin also inhibited DHA-induced degradation of newly synthesized apoB, assessed in a pulse-chase analysis using [^{35}S]methionine for metabolic labeling.

Discussion

The present results establish that the treatment of hepatic cells with Ω-3 fatty acids induces the degradation of newly synthesized apoB through a process that is distinct from either the ERAD/proteasome pathway or re-uptake. Thus, it is a third degradative "threat" to nascent apoB. This third threat falls somewhere between the other processes, which are at the two temporal extremes of the secretory pathway. The discussion to follow will focus on the intrinsic characteristics of this third process and its potential to regulate apoB-lipoprotein production in different physiologic and pathophysiologic states.

What is the substrate of Ω-3 fatty acid-induced degradation? Recent data (summarized in (3)) support the model that a small apoB-containing lipoprotein of HDL density is the "primordial" particle representing the completion of the MTP-dependent phase of initial lipidation in the ER. These primordial particles can be secreted directly, in which case they appear in media in the HDL density range, or they can be further lipidated to VLDL density, and then secreted. It has also been shown that apoE associates with nascent VLDL particles after apoB does (39-41,45). Taken with recent evidence that apoE promotes VLDL triglyceride

production (46), this suggests that the association of nascent VLDL with apoE occurs during the conversion of primordial apoB-lipoproteins to more lipidated particles. Thus, the relative refractoriness of apoB-lipoproteins more dense than VLDL to degradation stimulated by DHA or EPA (**Figs. 1 and 2**) and the global effects of DHA and EPA on apoE and the other VLDL apoproteins (**Figs. 3 and 4**; **Table 1**) indicate that Ω-3 fatty acids act late in the pathway of lipoprotein assembly. The substrate, then, like that for re-uptake, is an assembled apoB-lipoprotein.

Where does the Ω-3 fatty acid-induced degradation of newly synthesized apoB occur? For ERAD and re-uptake, targeting of apoB to degradation takes place at the ER or plasma membrane, respectively. Based on the heparin data (**Fig. 9**), Ω-3 fatty acids do not exert their effects through a re-uptake process at the cell surface. In contrast to ERAD and re-uptake, Ω-3 fatty acid-induced proteolysis is a post-ER event, given the protection afforded to apoB by Brefeldin A treatment (**Fig. 10**). For convenience, the pathway of hepatic apoB degradation induced by Ω-3 fatty acids will be referred to as "post-ER proteolysis" or PERP.

What are the likely mechanisms involved in post-ER proteolysis of apoB? Certainly, proteases are present in post-ER compartments (47), as well as in the Golgi apparatus (e.g.,(48)). Moreover, Cartright *et al.* have reported that in subcellular fractions of rabbit hepatocytes there was a Golgi-related degradation of apoB, based on incubations *in vitro* with a variety of broad protease inhibitors (49). The signal that identifies $apoB_{100}$ or $apoB_{48}$ for post-ER proteolysis in the presence of Ω-3 fatty acids is unknown, but several features can be inferred from the data. First, the signal must be related to some metabolic effect(s) of Ω-3 fatty acids. Possibilities include incorporation of these unusual molecules into lipids, such as triglycerides, cholesteryl ester, and phospholipids, which then change the physical properties of cell membranes or nascent lipoproteins. Other possibilities include competition for fatty acylation of proteins, such as apoB (50,51), or incorporation into eicosanoids (52). Of interest, palmitoylation of apoB in the ER of McArdle cells is required for the proper intracellular sorting of lipoproteins to the Golgi (53).

Second, the signal for Ω-3 fatty acid-induced apoB degradation must be intrinsically related to large, buoyant lipoproteins. It is theoretically possible that the features responsible for targeting apoB-containing lipoproteins in cells incubated with Ω-3 fatty acids are established before the particles are fully lipidated or exit from the ER and serve to trigger the appropriate trafficking of the doomed substrate to further lipidation and then post-ER degradation.

Nevertheless, the simpler idea that apoB targeting for post-ER degradation occurs as a consequence of extensive lipidation is favored. In particular, the association of apoB with large amounts of Ω-3 fatty acid-enriched lipids may favor proteolysis. Because VLDL-phospholipid composition is remodeled in the Golgi (54) and Ω-3 fatty acids are preferentially incorporated into phospholipids (e.g., (42)), perhaps it is by changing the physical properties of the lipoprotein surface that DHA or EPA influences the fate of apoB. The conformation of apoB may be altered, making it more susceptible to a protease, or the affinity of the nascent particle for chaperones, receptors, or other apoproteins may change, thereby promoting degradation. Third, interactions within the secretory pathway between nascent lipoproteins and their receptors, as has been proposed (14) and (16), could conceivably play a role, but would require novel effects of ligand-receptor binding. For example, adsorption of apoE by buoyant $apoB_{48}$-particles could allow binding to intracellular LDL receptors or HSPGs, but extra-lysosomal degradation would then be an unexpected consequence.

What is the general significance of Ω-3 fatty acid induced degradation? This question should be considered in the context of two other metabolic perturbations that induce apoB degradation, namely, choline deficiency and stimulation by insulin. Vance and colleagues (23,55,56) have shown that severe restriction of phospholipid synthesis by choline deficiency increases degradation of newly synthesized apoB in rat hepatocytes and hepatoma cells. In cell fractionation studies, these authors observed that choline-deficient hepatocytes exhibited nearly normal levels of apoB in the ER, but abnormally low levels in the Golgi apparatus. Furthermore, the most lipidated, buoyant lipoproteins were most affected by choline deficiency, regardless

of the length of the apoB molecule. Thus, degradation of apoB degradation induced by Ω-3 fatty acids and by choline deficiency share key, unusual features: degradation in a post-ER, pre-secretory compartment, and a preference for more lipidated apoB particles.

Another well-characterized metabolic perturbation that enhances hepatic apoB degradation is treatment of primary rat hepatocytes with insulin. Similar to the results for Ω-3 fatty acid-induced degradation, insulin treatment stimulates a degradative process that is 1) preferential for large, buoyant lipoproteins (24,57); 2) resistant to proteasome inhibitors (J. Sparks, unpublished studies); 3) post-ER (based on Brefeldin A studies; (43); and 4) inhibited by wortmannin (43,44). Again, these key, unusual characteristics resemble the degradation induced by Ω-3 fatty acids. The shared characteristics of the degradation induced by Ω-3 fatty acids, choline deficiency, and insulin suggest that PERP may be a general pathway for destruction of buoyant apoB-lipoproteins.

Based on many experimental studies, it has been generally concluded that the major determinant of hepatic apoB secretion *in vivo* is pre-secretory degradation (for recent reviews, see (2,3,24)). Which of these three "threats"- ERAD, re-uptake, and PERP- is likely to play important physiologic and pathophysiologic roles? ERAD can be provoked by MTP deficiency (as reviewed in (4)) or inhibition (11,12), and there is evidence that basal MTP lipid transfer activity may (e.g., (58)) or may not (e.g., (59)) be a limiting factor in apoB-lipoprotein assembly. At present, there is no direct evidence that regulation of MTP expression under *physiological* conditions significantly affects apoB output *in vivo*. Consistent with this are recent studies which have found that the addition of proteasome inhibitors to hepatocytes from ad lib fed rats or mice produced no increase in apoB output (J.D. Sparks, X. Wu, and E. Fisher, unpublished).

ERAD provoked by lipid deficiency seems a poor candidate to make significant contributions to apoB degradation under normal physiological conditions, because the phenomenon is observed primarily in the livers of animals fasted for a prolonged period of time (60) or in cell culture models of starvation (e.g., (61)), in

which the fatty acid concentration needs to be maintained below a level to which human plasma free fatty acid levels do not normally fall. Furthermore, the apoC-III transgenic mouse provides a specific example of increased fatty acid availability *in vivo,* but without any increase in hepatic apoB secretion (62).

Thus, the two other "threats", re-uptake and PERP, are more plausible modulators of apoB degradation *in vivo*. Unlike ERAD, re-uptake and PERP both occur in normal primary hepatocytes. In the whole animal, the diurnal variation in hepatic cholesterol biosynthesis could affect LDL receptor activity and, consequently, re-uptake. In addition, it has recently been reported that LDL-receptor knockout mice have increased VLDL production (16), consistent with an impairment of re-uptake (see also (63)). Altered expression of bridging molecules, leading to enhanced re-uptake via HSPGs, may account for some hypolipidemic effects of fibric acid derivatives *in vivo* (17,64). Regarding PERP, the consistent clinical finding of lowered VLDL levels in human subjects consuming a Ω-3 fatty acid-enriched diet (26), plus kinetic studies *in vivo* (65) that demonstrated decreased hepatic VLDL production with Ω-3 fatty acid consumption, are consistent with a pathway that preferentially eliminates large, buoyant lipoproteins within the liver of whole organisms. Furthermore, the reduced plasma triglyceride levels in choline-deficient rats and the finding that hepatocytes isolated from these rats exhibited increased apoB degradation and decreased VLDL secretion (23,55) demonstrate that hepatic post-ER proteolysis of apoB was induced in the intact animal, although under an extreme dietary condition. Finally, it was recently demonstrated in metabolic studies *in vivo* (66) that the acute post-prandial rise in insulin levels is associated with decreased hepatic VLDL production *in vivo*, exactly as expected from studies of PERP *in vitro*. Thus, there is ample evidence to support a role for PERP *in vivo* to modulate VLDL production in response to various metabolic stimuli.

PERP also provides an attractive hypothesis to explain the underlying basis for the overproduction of VLDL particles in familial combined hyperlipidemia (FCHL), syndrome X, and the metabolic syndrome (for recent reviews, see (67,68)). These disorders, which all confer an increased risk of coronary artery

disease, may originate in part from insulin resistance. Because PERP appears to be induced *in vitro* and *in vivo* by insulin, it should be inhibited by insulin resistance, thereby resulting in VLDL overproduction. Consistent with this model, insulin-resistant animals were recently reported to exhibit decreased degradation of newly synthesized apoB (69).

In conclusion, the elucidation of the mechanisms by which apoB is targeted to PERP and the identification of the protease(s) in this process should illuminate an important regulatory mechanism for hepatic lipoprotein production in both normal and pathophysiological states and provides a new target for pharmacologic intervention.

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What is claimed is:

1. A method of treating a patient with a cardiovascular disorder, comprising administering a therapeutically effective amount of a compound to said patient, wherein said compound acts for a period of time to lower plasma concentrations of apoB or apoB-containing lipoproteins by stimulating a pathway for apoB degradation that is independent of ERAD or cell/surface re-uptake or that occurs in a post-ER compartment or compartments.

2. The method of **Claim 1**, wherein the cardiovascular disorder is one of the group of angina, atherosclerosis, restenosis, claudication, unstable angina, stroke, transient ischemic attacks, coronary artery disease, peripheral vascular disease, cerebral vascular disease, and endothelial dysfunction.

3. A method of treating a patient with a metabolic disorder or syndrome, comprising administering a therapeutically effective amount of a compound to said patient, wherein said compound acts for a period of time to lower plasma concentrations of apoB or apoB-containing lipoproteins by stimulating a pathway for apoB degradation that is independent of ERAD or cell/surface re-uptake or that occurs in a post-ER compartment or compartments.

4. The method of **Claim 3**, wherein the metabolic disorder or syndrome is one of the group of hypercholesterolemia, hyperlipidemia, hyper lipoproteinemia, familial hyperlipidemia, syndrome X, insulin resistance syndromes, diabetes, and metabolic disorders associated with vascular disease.

5. A method for screening pharmaceutical compounds, wherein said compounds stimulate a pathway for pre-secretory apoB degradation independent of ERAD or cell/surface re-uptake or said compounds stimulate a pathway for pre-secretory apoB degradation that occurs in a post-ER compartment or compartments, said screening requiring the steps of applying at least one candidate compound to a

hepatic cell line, thereby causing reduced apoB secretion to said hepatic cell line and assaying for said apoB secretion.

6. The method of **Claim 5**, further comprising a screen for reduced apoB secretion from cultured hepatic cells followed by a step to identify the reduction as independent of ERAD or cell/surface re-uptake or as occurring in a post-ER compartment or compartments.

7. The method of **Claim 5**, further comprising a screen for reduced apoB secretion from cultured hepatic cells followed by a step to identify the reduction as dependent on PI-3 kinase.

8. The method of **Claim 5**, in which said pharmaceutical compounds are selected from the group consisting of marine oils, PPAR agonists, PPAR antagonists, nicotinic acid, cholesterol synthesis inhibitors, lipid synthesis inhibitors, inducers of Golgi-associated hydrolases, inducers of a hydrolase in a post-ER compartment, cytokine receptor agonists, cytokine receptor antagonists, inducers of PI-3 kinase, and analogues and derivatives thereof.

9. A method for screening genes, wherein said genes participate in a pathway for pre-secretory apoB degradation independent of ERAD or cell/surface re-uptake or that occurs in a post-ER compartment or compartments, said screening occurring by at least one of the following:

transfection of a candidate gene or antisense construct to a hepatic cell line and assay for apoB recovery;

transfection of a library construct to a hepatic cell line and assay for apoB recovery; and

use of an knock-out or modified animal wherein primary hepatocytes have altered apoB production and apoB degradation in said animal is compared with apoB degradation in wild-type hepatocytes of a normal or modified animal.

10. A method of treating a patient with a cardiovascular disorder, comprising administering a therapeutically effective amount of a compound to said patient, wherein said compound acts for a period of time to lower plasma concentrations of apoB or apoB-containing lipoproteins by stimulating a pathway for apoB degradation that occurs in the Golgi apparatus.

11. The method of **Claim 10**, wherein the cardiovascular disorder is one of the group of angina, atherosclerosis, restenosis, claudication, unstable angina, stroke, transient ischemic attacks, coronary artery disease, peripheral vascular disease, cerebral vascular disease, and endothelial dysfunction.

12. A method of treating a patient with a metabolic disorder or syndrome, comprising administering a therapeutically effective amount of a compound to said patient, wherein said compound acts for a period of time to lower plasma concentrations of apoB or apoB-containing lipoproteins by stimulating a pathway for apoB degradation that occurs in the Golgi apparatus.

13. The method of **Claim 12**, wherein the metabolic disorder or syndrome is one of the group of hypercholesterolemia, hyperlipidemia, hyper □□lipoproteinemia, familial hyperlipidemia, syndrome X, insulin resistance syndromes, diabetes, and metabolic disorders associated with vascular disease.

14. A method for screening pharmaceutical compounds, wherein said compound stimulates a pathway for pre-secretory apoB degradation that occurs in the Golgi apparatus said screening requiring the steps of applying at least one candidate compound to a hepatic cell line, thereby causing reduced apoB secretion to said hepatic cell line and assaying for said apoB secretion.

15. The method of **Claim 14**, further comprising a screen for reduced apoB secretion from cultured hepatic cells followed by a step to identify the reduction as occurring in the Golgi apparatus.

16. The method of **Claim 14**, further comprising a screen for reduced apoB secretion from cultured hepatic cells followed by a step to identify the reduction as enhanced by monensin.

17. The method of **Claim 14**, in which said pharmaceutical compounds are selected from the group consisting of marine oils, PPAR agonists, PPAR antagonists, nicotinic acid, cholesterol synthesis inhibitors, lipid synthesis inhibitors, inducers of a hydrolase in a post-ER compartment, inducers of Golgi-associated hydrolases, cytokine receptor agonists, cytokine receptor antagonists, inducers of PI-3 kinase, and analogues and derivatives thereof.

18. A method for screening genes, based on their participation in a pathway for pre-secretory apoB degradation that occurs in the Golgi apparatus said screening occurring by at least one of the following:

transfection of a candidate gene or antisense construct to a hepatic cell line and assay for apoB recovery;

transfection of a library construct to a hepatic cell line and assay for apoB recovery; and

use of an knock-out or modified animal wherein primary hepatocytes have altered apoB production and apoB degradation in said animal is compared with apoB degradation in wild-type hepatocytes of a normal or modified animal.

19. A method of treating a patient with a cardiovascular disorder, comprising administering a therapeutically effective amount of a compound to said patient, wherein said compound acts for a period of time to lower plasma concentrations of apoB or apoB-containing lipoproteins by stimulating a pathway for apoB degradation that occurs in the intermediate compartment.

20. The method of **Claim 19**, wherein the cardiovascular disorder is one of the group of angina, atherosclerosis, restenosis, claudication, unstable angina,

stroke, transient ischemic attacks, coronary artery disease, peripheral vascular disease, cerebral vascular disease, and endothelial dysfunction.

21. A method of treating a patient with a metabolic disorder or syndrome, comprising administering a therapeutically effective amount of a compound to said patient, wherein said compound acts for a period of time to lower plasma concentrations of apoB or apoB-containing lipoproteins by stimulating a pathway for apoB degradation that occurs in the intermediate compartment.

22. The method of **Claim 21**, wherein the metabolic disorder or syndrome is one of the group of hypercholesterolemia, hyperlipidemia, hyper lipoproteinemia, familial hyperlipidemia, syndrome X, insulin resistance syndromes, diabetes, and metabolic disorders associated with vascular disease.

23. A method for screening pharmaceutical compounds, wherein said compound stimulates a pathway for pre-secretory apoB degradation that occurs in the intermediate compartment said screening requiring the steps of applying at least one candidate compound to a hepatic cell line, thereby causing reduced apoB secretion to said hepatic cell line and assaying for said apoB secretion.

24. The method of **Claim 23**, further comprising a screen for reduced apoB secretion from cultured hepatic cells followed by a step to identify the reduction as occurring in the intermediate compartment.

25. The method of **Claim 23**, in which said pharmaceutical compounds are selected from the group consisting of marine oils, PPAR agonists, PPAR antagonists, nicotinic acid, cholesterol synthesis inhibitors, lipid synthesis inhibitors, inducers of a hydrolase in a post-ER compartment, inducers of Golgi-associated hydrolases, cytokine receptor agonists, cytokine receptor antagonists, inducers of PI-3 kinase, and analogues and derivatives thereof.

26. A method for screening genes, based on their participation in a pathway for pre-secretory apoB degradation that occurs in the intermediate compartment said screening occurring by at least one of the following:

transfection of a candidate gene or antisense construct to a hepatic cell line and assay for apoB recovery;

transfection of a library construct to a hepatic cell line and assay for apoB recovery; and

use of an knock-out or modified animal wherein primary hepatocytes have altered apoB production and apoB degradation in said animal is compared with apoB degradation in wild-type hepatocytes of a normal or modified animal.

27. A method of treating a patient with a cardiovascular disorder, comprising administering a therapeutically effective amount of a compound to said patient, wherein said compound acts for a period of time to lower plasma concentrations of apoB or apoB-containing lipoproteins by stimulating a pathway for apoB degradation that occurs post-Golgi apparatus and independent of cell/surface re-uptake.

28. The method of **Claim 27**, wherein the cardiovascular disorder is one of the group of angina, atherosclerosis, restenosis, claudication, unstable angina, stroke, transient ischemic attacks, coronary artery disease, peripheral vascular disease, cerebral vascular disease, and endothelial dysfunction.

29. A method of treating a patient with a metabolic disorder or syndrome, comprising administering a therapeutically effective amount of a compound to said patient, wherein said compound acts for a period of time to lower plasma concentrations of apoB or apoB-containing lipoproteins by stimulating a pathway for apoB degradation that occurs post-Golgi apparatus and independent of cell/surface re-uptake.

30. The method of **Claim 29**, wherein the metabolic disorder or syndrome is one of the group of hypercholesterolemia, hyperlipidemia, hyper

lipoproteinemia, familial hyperlipidemia, syndrome X, insulin resistance syndromes, diabetes, and metabolic disorders associated with vascular disease.

31. A method for screening pharmaceutical compounds, wherein said compound stimulates a pathway for pre-secretory apoB degradation that occurs post-Golgi apparatus and independent of cell surface re-uptake said screening requiring the steps of applying at least one candidate compound to a hepatic cell line, thereby causing reduced apoB secretion to said hepatic cell line and assaying for said apoB secretion.

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32. The method of **Claim 31**, further comprising a screen for reduced apoB secretion from cultured hepatic cells followed by a step to identify the reduction as occurring post-Golgi apparatus and independent of cell/surface re-uptake.

33. The method of **Claim 31**, in which said pharmaceutical compounds are selected from the group consisting of marine oils, PPAR agonists, PPAR antagonists, nicotinic acid, cholesterol synthesis inhibitors, lipid synthesis inhibitors, inducers of a hydrolase in a post-ER compartment, inducers of Golgi-associated hydrolases, cytokine receptor agonists, cytokine receptor antagonists, inducers of PI-3 kinase, and analogues and derivatives thereof.

34. The method of **Claim 5**, in which said pre-secretory apoB degradation involves a lipoprotein receptor.

35. The method of **Claim 34**, in which said lipoprotein receptor is one of the group of an LDL receptor, LDL receptor family members, an LDL receptor-related protein, heparan sulfate proteoglycans, scavenger receptors class A, scavenger receptors class B, any other scavenger receptor, and any calcium-dependent lipoprotein receptor.

36. The method of **Claim 5**, in which said pharmaceutical compounds are at least one of the group of agents that mimic choline deficiency; agents that block lipid transfer or exchange involving lipoproteins; agents that block phospholipid synthesis, transfer, hydrolysis, or exchange; agents that mimic insulin action; insulin; agents that stimulate the expression of a lipoprotein receptor; and agents that stimulate the expression of a bridging molecule.

37. The method of **Claim 36**, in which said bridging molecule facilitates the binding of a lipoprotein to a lipoprotein receptor.

38. The method of **Claim 36**, in which said bridging molecule is selected from the group consisting of apolipoprotein-E, lipoprotein lipase, hepatic lipase, a defensin, and any molecule with a hydrophobic face and a cationic face.

39. The method of **Claim 5**, in which said pre-secretory apoB degradation is sensitive to EGTA.

40. The method of **Claim 5**, in which said pre-secretory apoB degradation requires calcium.

41. The method of **Claim 5**, in which said pre-secretory apoB degradation requires a calcium-dependent protease.

42. The method of **Claim 5**, in which said pre-secretory apoB degradation requires a calcium-dependent lipoprotein receptor.

43. The method of **Claim 5**, in which said pre-secretory apoB degradation is independent of MTP inhibition, active proteosomes, cell-surface LDL receptors, cell-surface heparan sulfate proteoglycans, or

functioning lysosomes.

44. The method of **Claim 5**, in which said pre-secretory apoB degradation destroys a target particle that is a large and relatively mature lipoprotein.

45. The method of **Claim 44**, in which said large and relatively mature lipoprotein contains substantial amounts of lipid and other apoproteins, said apoproteins selected from the group consisting of apoE and apoCs.

46. The method of **Claim 5**, in which said pre-secretory apoB degradation is defective in a disorder selected from the group consisting of familial combined hyperlipidemia, the metabolic syndrome, syndrome-X, insulin resistance, and any condition associated with apoB oversecretion.

47. The method of **Claim 5**, in which said pre-secretory apoB degradation involves a chaperone molecule.

48. The method of **Claim 5**, in which said pre-secretory apoB degradation involves an integrin molecule.

49. The method of **Claim 48**, in which said integrin molecule has a cytosolic extension that binds calcium.

50. The method of **Claim 47**, in which said chaperone molecule is selected from the group consisting of members of the families of hsp70, 90, and 60.

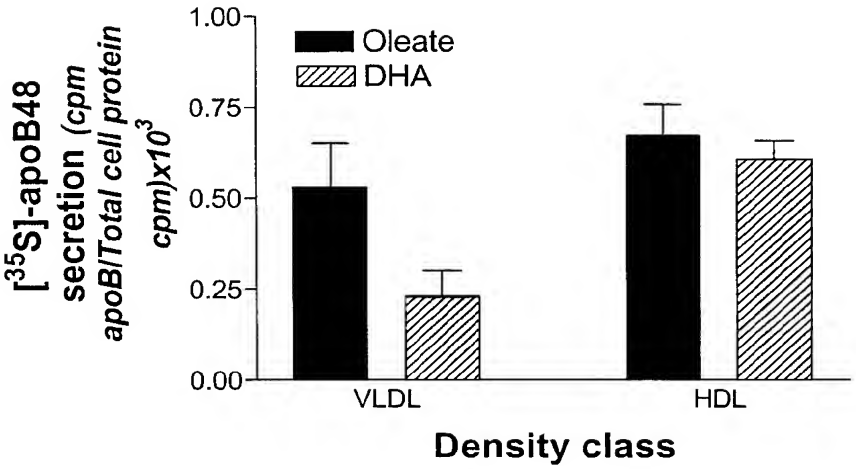
[35S]-apoB48 secretion (cpm apoB/Total cell protein cpm)x10^3
1.00
0.75
0.50
0.25
0.00
Oleate
DHA
VLDL
HDL
Density class

Fig. 1

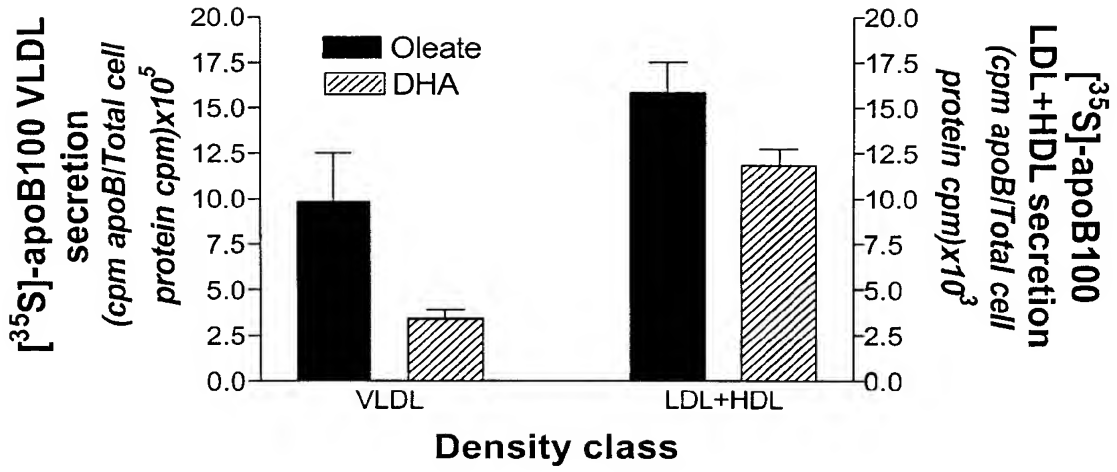
[35S]-apoB100 VLDL secretion (cpm apoB/Total cell protein cpm)x10^5
[35S]-apoB100 LDL+HDL secretion (cpm apoB/Total cell protein cpm)x10^3
Oleate
DHA
20.0
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
VLDL
LDL+HDL
Density class

Fig. 2

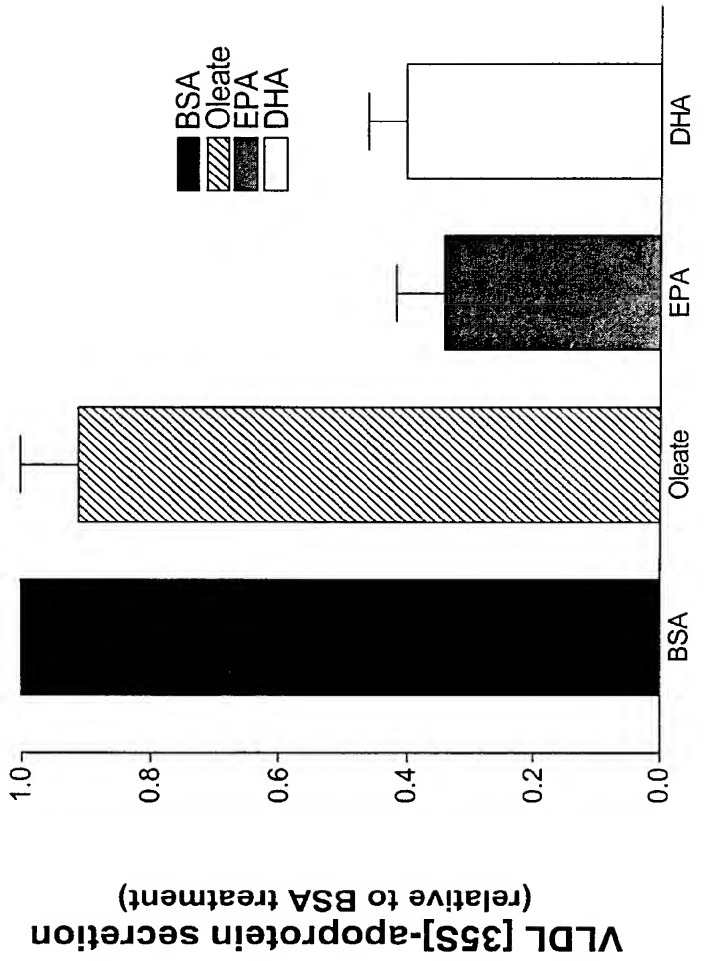
VLDL [35S]-apoprotein secretion
(relative to BSA treatment)
1.0
0.8
0.6
0.4
0.2
0.0
BSA
Oleate
EPA
DHA
BSA
Oleate
EPA
DHA

Fig. 3

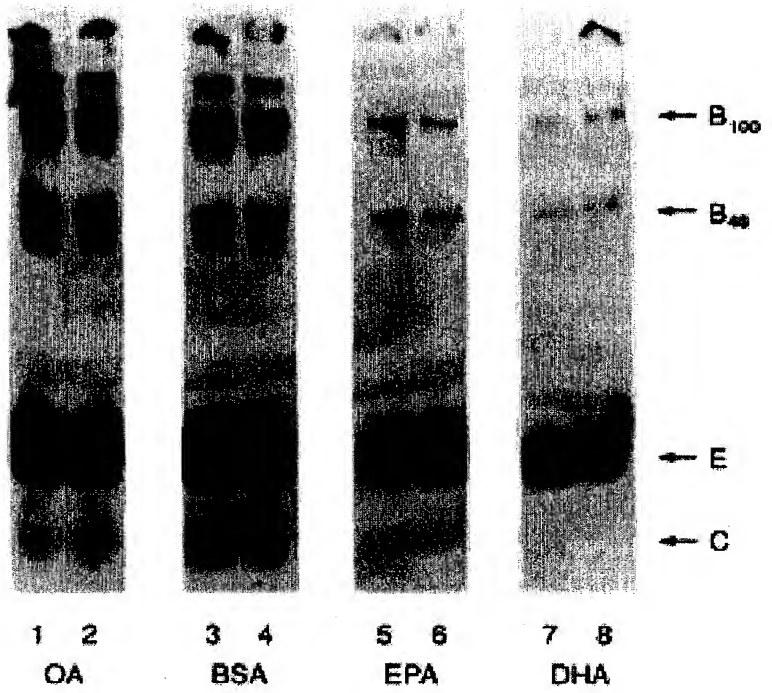

Fig. 4

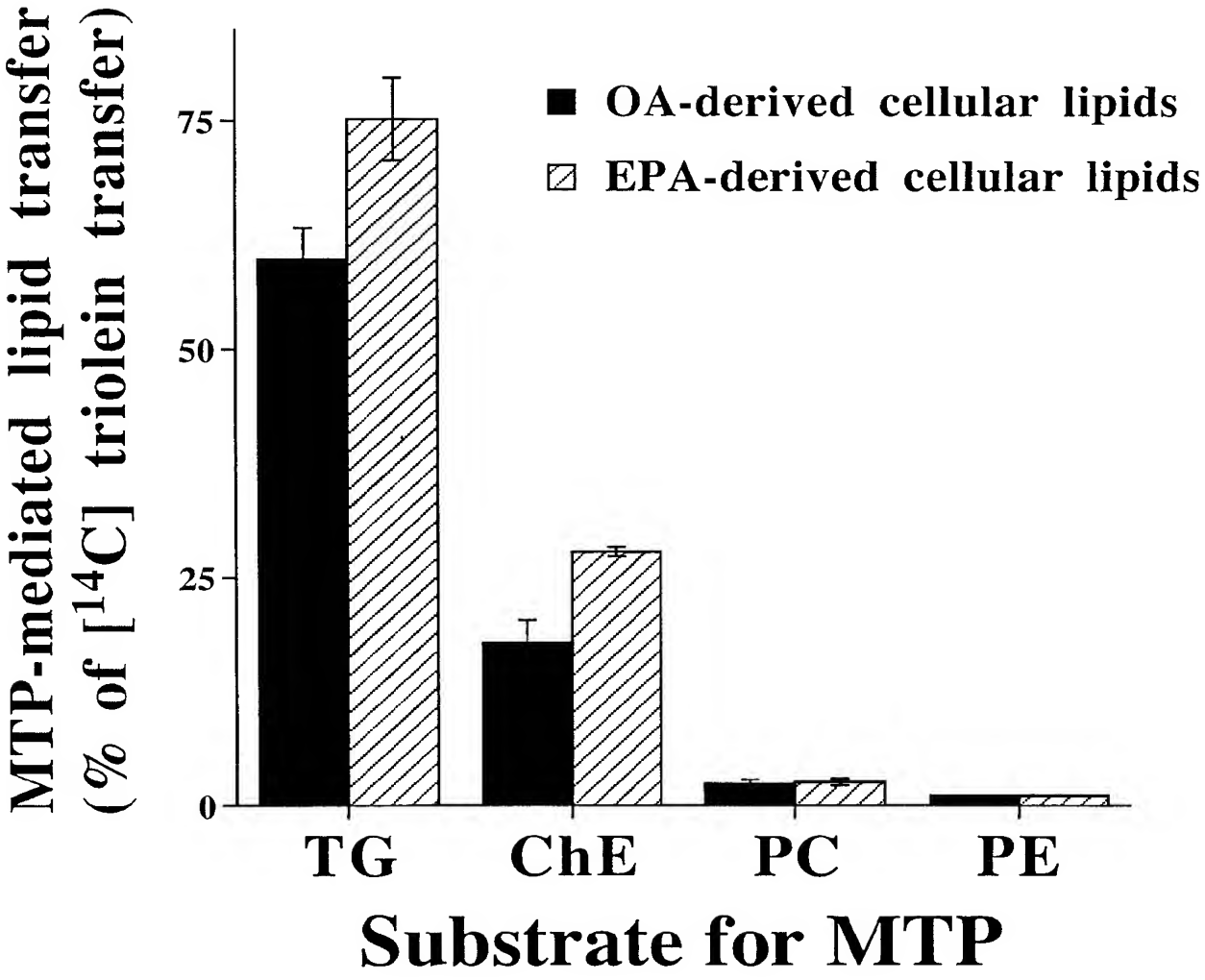
MTP-mediated lipid transfer
(% of [14C] triolein transfer)
75
50
25
0
OA-derived cellular lipids
EPA-derived cellular lipids
TG
ChE
PC
PE
Substrate for MTP

Fig. 5

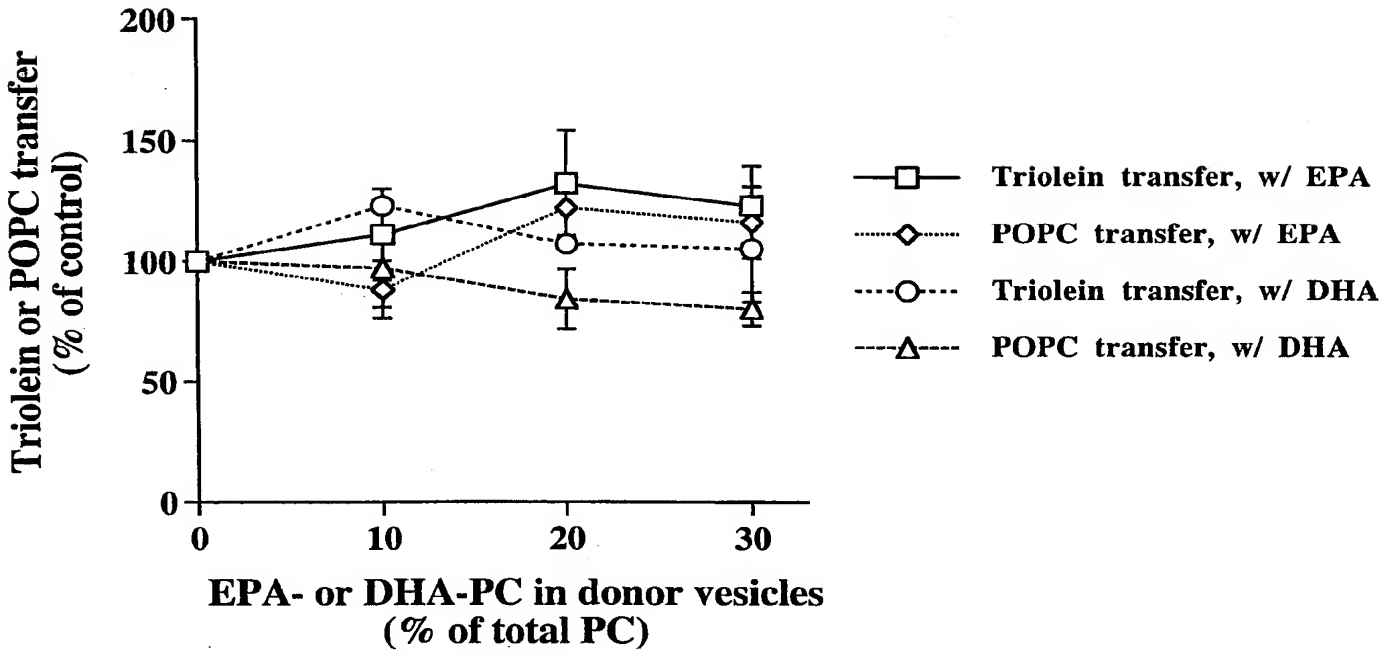
Triolein or POPC transfer
(% of control)
200
150
100
50
0
0
10
20
30
EPA- or DHA-PC in donor vesicles
(% of total PC)
Triolein transfer, w/ EPA
POPC transfer, w/ EPA
Triolein transfer, w/ DHA
POPC transfer, w/ DHA

Fig. 6

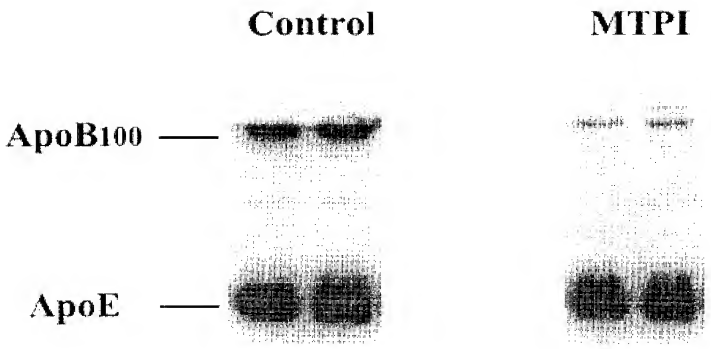

Fig. 7

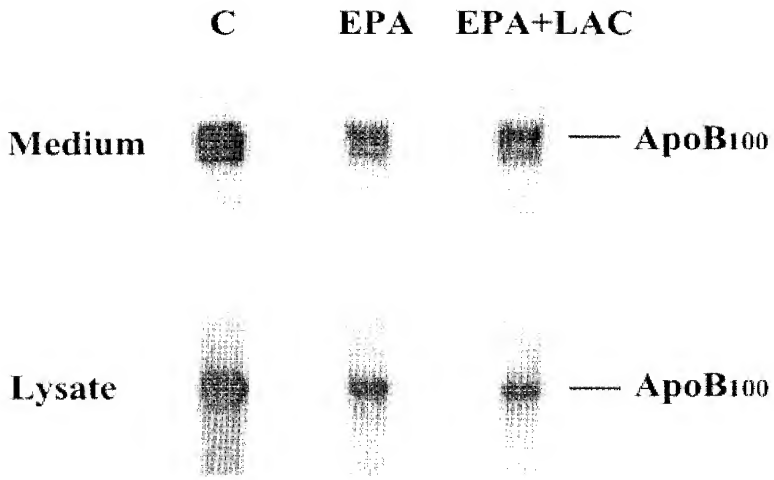

Fig. 8

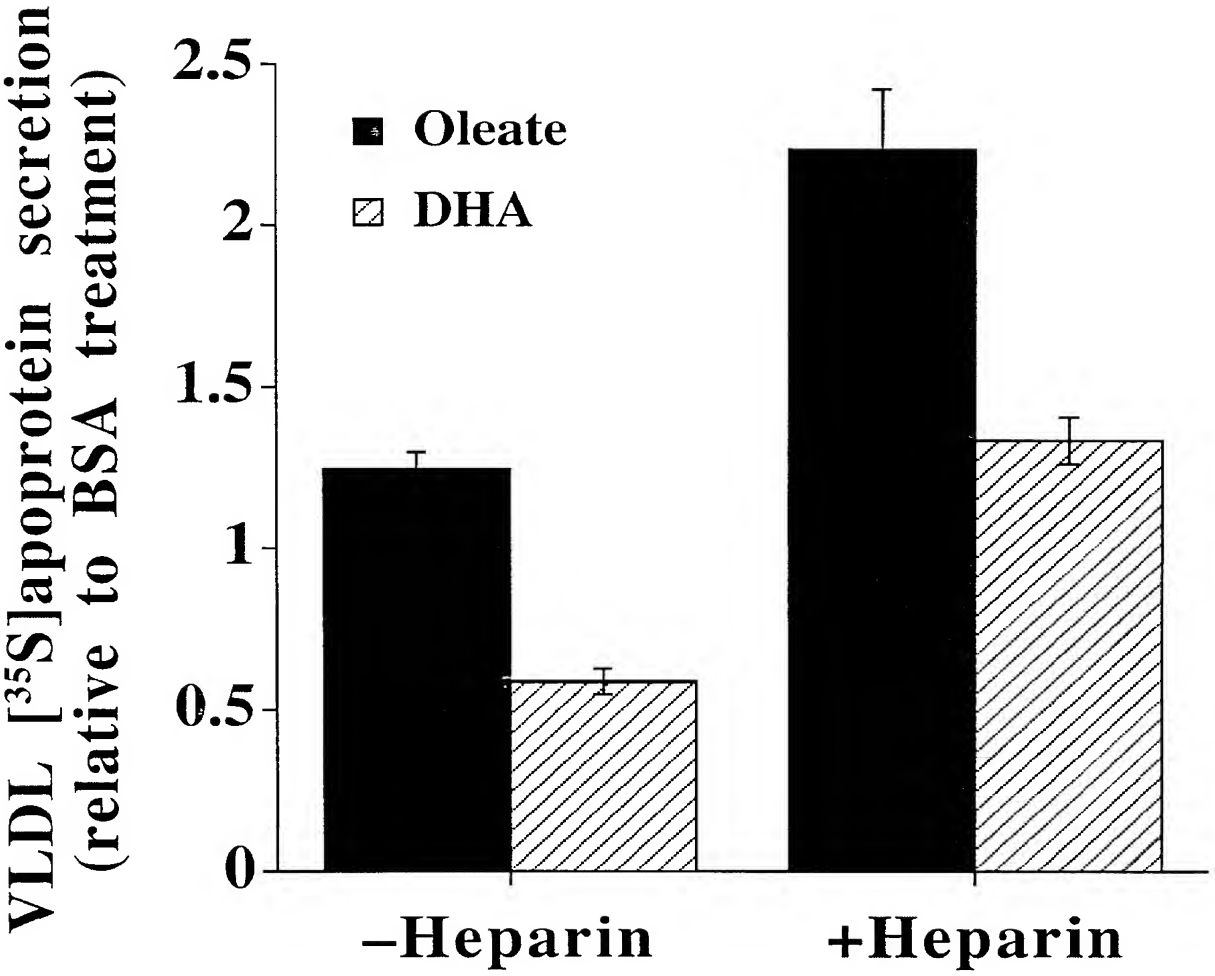
VLDL [35S]apoprotein secretion
(relative to BSA treatment)
2.5
2
1.5
1
0.5
0
Oleate
DHA
–Heparin
+Heparin

Fig. 9

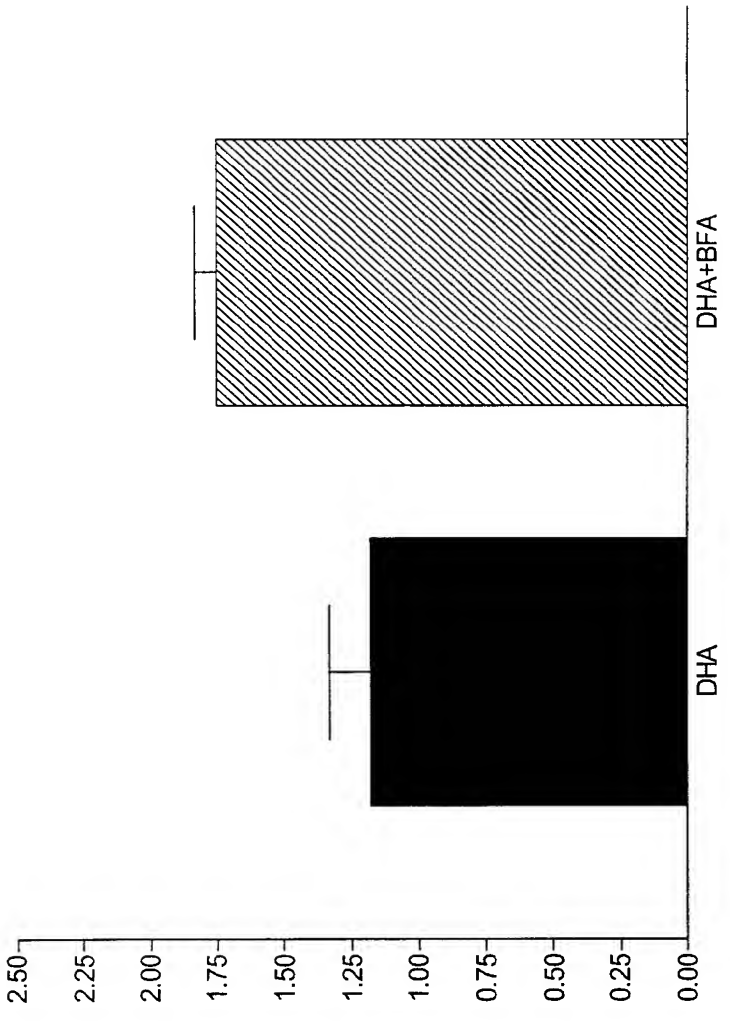
DHA+BFA
DHA
2.50
2.25
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
apoB100 phosphorimager units
(normalized to total protein cpm)

Fig. 10

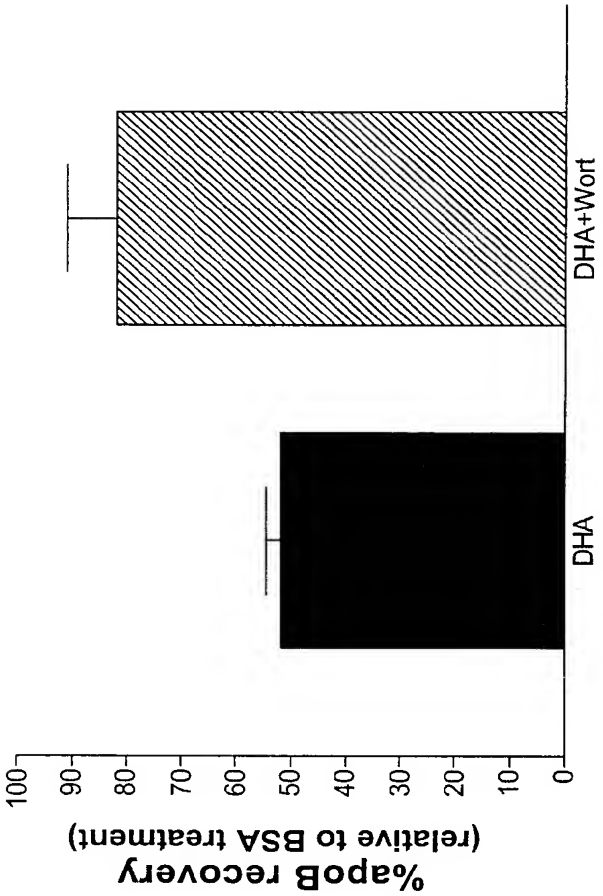
%apoB recovery
(relative to BSA treatment)
100
90
80
70
60
50
40
30
20
10
0
DHA
DHA+Wort

Fig. 11

Two known mechanisms for destruction of newly synthesized hepatic apoB: ERAD/Proteasome and re-uptake

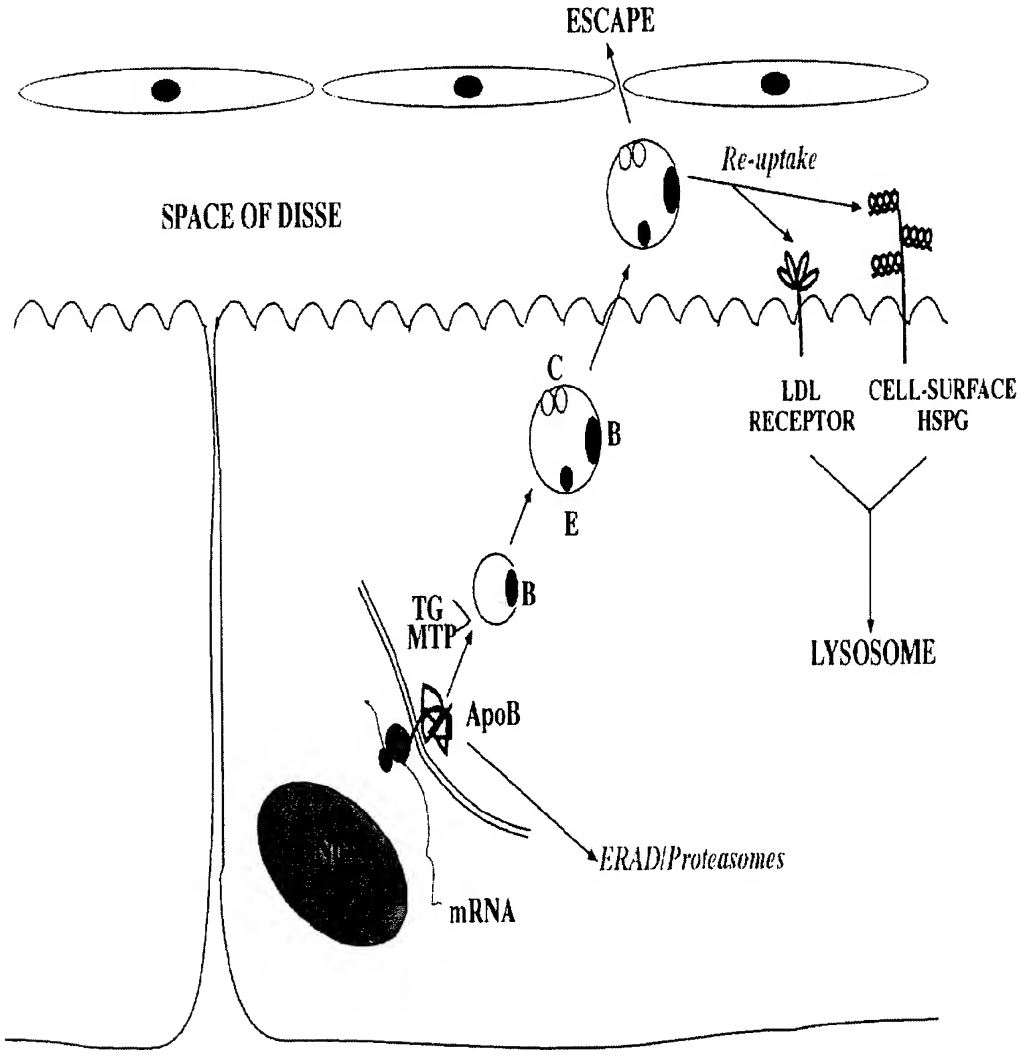

Fig. 12

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/29699

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :A61K 31/56
US CL : 514/178

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/178

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 4,920,115 A (NESTLER et al.) 24 April 1990, see the entire document.	1-50

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 27 DECEMBER 2000	Date of mailing of the international search report 26 FEB 2001
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